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Rapid and highly automated determination of morphine and morphine glucuronides in plasma by on-line solid-phase extraction and column liquid chromatography

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Abstract

A high-performance liquid chromatography (HPLC) method has been developed for the determination of morphine and its main metabolites, morphine-6-glucuronide (M-6-G) and morphine-3-glucuronide (M-3-G), in plasma or cerebrospinal fluid. Samples were extracted using on-line solid-phase extraction followed by reversed-phase HPLC with fluorescence detection. Recoveries of 20 ng morphine and morphine glucuronides in plasma were over 95%. The limit of detection using 400 μl of a biological matrix was 0.85, 3.4 and 1.0 ng/ml of M-3-G, M-6-G and morphine, respectively. Inter- and intra-day assay precision was better than 10%. The main advantages of the present described method are increased recoveries (>95%) and a high degree of automation allowing a high speed in routine analysis. The time required for the fully automated analysis of one sample was less than 26 min.

1. Introduction

Morphine glucuronides belong to the major metabolites of morphine. The marked analgesic potency of morphine-6-glucuronide [1–3] has highlighted the importance of determining the behaviour of both morphine and morphine metabolites in vivo [4–7].

Previous approaches used for the determination of morphine and its metabolites in a biological matrix have included radioimmunoassay (RIA) [8,9], gas chromatography (GC) with electron-capture detection [10] or mass spectrometry [11] and high-performance liquid chromatography (HPLC) with electrochemical detection (ED) [12–15], UV detection [16,17] and fluorescence detection [18–20]. Assays using only ED, however, are unable to detect M-3-G as it lacks an oxidizable phenolic hydroxyl group. A major problem associated with HPLC is the need of sophisticated extraction procedures to separate the compounds of interest from a biological matrix prior to analysis. Such extraction procedures introduced during the last years made use of either liquid–liquid extraction schemes [21,22] or solid-phase extraction methods [19,23–28]. These extraction schemes have, however, the disadvantage that they require laborious and time-consuming manual treatment of each individual sample and in most cases relatively large volumes of biological matrix.

In this study a method is described which

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allows fully automated solid-phase extraction and on-line analysis of morphine and morphine glucuronides in small samples of plasma or cerebrospinal fluid. Both sample extraction on a pre-column and elution onto the analytical column were performed automatically and concomitantly using conventional HPLC-equipment in conjunction with an OSP-2 on-line sample preparator from Merck.

2. Experimental

2.1. Materials

Morphine-HCl (M) was from Sintetica (Mendrisio, Switzerland). Morphine-6β-glucuronide · $2H_2O$ (M-6-G) was from Mundipharma (Basel, Switzerland). Morphine-3β-glucuronide (M-3-G) was from Ultrafine Chemicals (Manchester, UK). Solvents were HPLC-grade (LiChrosolv) and obtained from Merck (Darmstadt, Germany). All other chemicals were from commercial sources and of analytical grade.

2.2. Instrumentation

The Merck HPLC-system consisted of two pumps (Models L-6200 and L-6000) combined with a solvent selector, an AS-2000 autosampler, an on-line sample preparator (Model OSP-2) and a variable-wavelength fluorescence detector (Model F-1050, all Merck-Hitachi). Data acquisition and analysis were performed using a personal computer and Perkin-Elmer-Nelson (UK) interfaces and software (Turbochrom, version 2.0).

2.3. Columns

LiChrosphere 60 RP-select B cartridges (4 mm × 4 mm I.D.; particle size 10 μm, Merck, Darmstadt) were used for the OSP-2. LiChrosphere 60RP-select B (250 mm × 4 mm I.D.; particle size 5 μm) combined with a 60 RP-select B guard column (4 mm × 4 mm I.D.; particle size 5 μm; both Merck) was used as analytical column.

2.4. Sample preparation

Stock solutions were prepared by dissolving morphine, M-3-G and M-6-G in water followed by an appropriate dilution using plasma or cerebrospinal fluid, respectively. Samples were kept at room temperature during analysis. Aliquots of 100 to 400 μl were injected using an autosampler AS-2000 (Merck). The drug and its main metabolites were separated from the organic matrix by automated on-line solid-phase extraction using the sample preparator OSP-2 (Merck). This device, which is described in detail in Refs. [29–31], allows on-line extraction of a sample while the previously extracted sample on the previous cartridge is eluted onto the analytical column. A new OSP-2 cartridge was used for each analysis.

2.5. HPLC conditions

Samples were loaded for extraction onto cartridges using 100 m*M* ammonium sulfate, adjusted for pH 9.3 using 25% NH_3 in water (sample loading buffer D). The mobile phase used for desorption from the cartridge and elution onto the analytical column consisted of a linear gradient (4–12% with respect to acetonitrile) formed by combination of 200 m*M* potassium phosphate, pH 3.0 (eluent A) and acetonitrile–200 m*M* potassium phosphate, pH 3.0, 20:80 (v/v) (eluent B). The high salt concentration is necessary to shift M-3-G towards higher retention times, necessary to obtain no interference with the system peak eluting at 4.6 min (Fig. 1). After analysis, the analytical column was regenerated using acetonitrile–methanol–water, 40:40:20 (v/v/v) (wash solvent C). New OSP-2 cartridges were pre-conditioned by the same wash procedure before use. Details are given in Table 1.

2.6. Detection

Opioids were detected by fluorescence detection (excitation wavelength 210 nm, emission wavelength 350 nm).

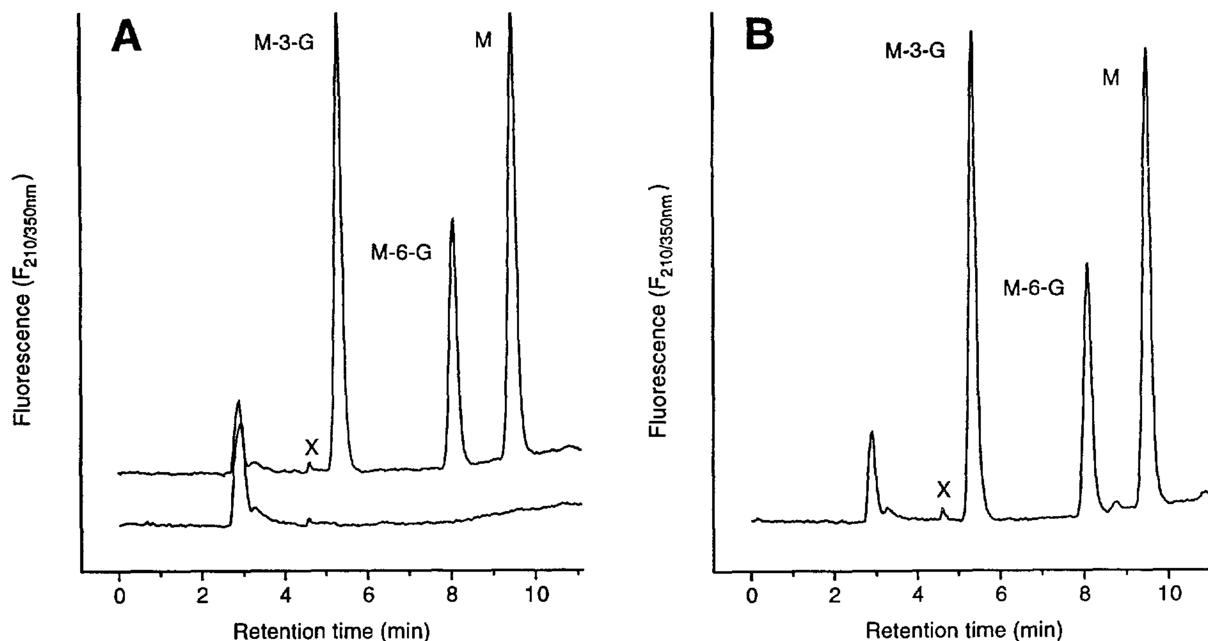

Fig. 1. Chromatograms showing (A, upper trace) extracted plasma and (B) cerebrospinal fluid spiked with 20 ng each of morphine-3-glucuronide (M-3-G), morphine-6-glucuronide (M-6-G) and morphine (M) (100 μl injection). X designates a system peak also present in an extracted blank (A, lower trace).

3. Results and discussion

3.1. Extraction

Recoveries were determined by comparing peak areas of directly injected standards in eluent A with peak areas of the respective compound after solid-phase extraction. Recoveries ± S.E.M. ($n = 16$) were found to be 95.9 ± 0.7, 99.2 ± 0.7 and 102.7 ± 0.7 for 20 ng each M-3-G, M-6-G and morphine in plasma, respectively. Plasma could be substituted by cerebrospinal fluid without any problems. In this case recoveries were 94.7 ± 0.3, 96.9 ± 0.4 and 99.8 ± 0.5 for 20 ng each M-3-G, M-6-G and morphine, respectively ($n = 4$).

3.2. Chromatography

Fig. 1 shows typical chromatograms of a plasma or a cerebrospinal fluid sample spiked with morphine and its glucuronides. Baseline separation of morphine, morphine-3-glucuronide and morphine-6-glucuronide is achieved with retention times of 5.3, 8.1 and 9.5 min, respectively. Calibration curves showed good linearity between peak areas and concentrations of M-3-G, M-6-G and morphine in plasma. Correlation coefficients (r^2) were always ($n = 4$ experiments) greater than 0.9999 for all compounds in the range of 0.5–100 ng. The accuracy of the assay was determined over the range 0.05–100 ng/ml. The results are given in Table 2.

3.3. Limit of detection

Limits of detection were defined as three times the signal-to-noise ratio. Thus minimal concentrations of morphine and morphine glucuronides which may be detected in plasma or cerebrospinal fluid depend on the sample volume applied to the system. Validation of this method was done using injection volumes of 100 μl. However, the autosampler we used for this study allowed injection volumes of up to 400 μl. There was no statistically significant decrease in signal between

Table 1
Experimental details

Time (min)	OSP-2				Solvent[c]	Pump L-6000, flow-rate (ml/min)	Pump L-6200		Comments
	Valve 1[a]	Valve 2[a]	Clamp[b]	Move			Eluent[c,d]	Flow-rate (ml/min)	
0.0	On	On	On	Off	D	0.50	60% B	0.8	Loading of cartridge; equilibration of analytical column
2.0	On	On	On	Off	D	0.74	100% C	1.2	
6.0	On	On	On	Off	D	1.22	100% C	1.2	
8.0	On	On	On	Off	D	1.40	20% B	1.2	
10.0	Off	On	On	Off	C	1.40	20% B	1.2	
10.1	Off	On	On	Off	C	1.00	20% B	1.2	
12.2	Off	Off	On	Off	C	1.00	20% B	1.2	Bypass of cartridges
12.3	Off	Off	Off	Off	C	1.00	20% B	1.2	Opening of clamp
12.4	Off	Off	Off	On	C	1.00	20% B	1.2	Move of cartridge to analytical side of OSP-2
12.5	On	Off	On	Off	C	1.00	20% B	1.2	Washing of cartridge
12.6	On	On	On	Off	C	1.00	20% B	0.8	Start of analytical separation and data aquisition
17.5	On	On	On	Off	D	1.00	36% B	0.8	Pre-conditioning of cartridge
20.5	On	On	On	Off	D	1.00	45% B	0.8	
20.6	On	On	On	Off	D	0.50	46% B	0.8	
25.0	On	On	On	Off	D	0.50	60% B	0.8	Start of next cycle

[a] Off = bypass of cartridge.
[b] Off = cartridge holding clamp open.
[c] Solvents: A is 200 m*M* potassium phosphate, pH 3.0; B is acetonitrile–200 m*M* potassium phosphate, pH 3.0, 20:80 (v/v); C is wash solvent, acetonitrile–methanol–water 40:40:20 (v/v/v); D is sample loading buffer, 100 m*M* ammonium sulfate, pH 9.3.
[d] Fractional flow of the indicated eluent. Difference between indicated percentage and 100% is eluent A.

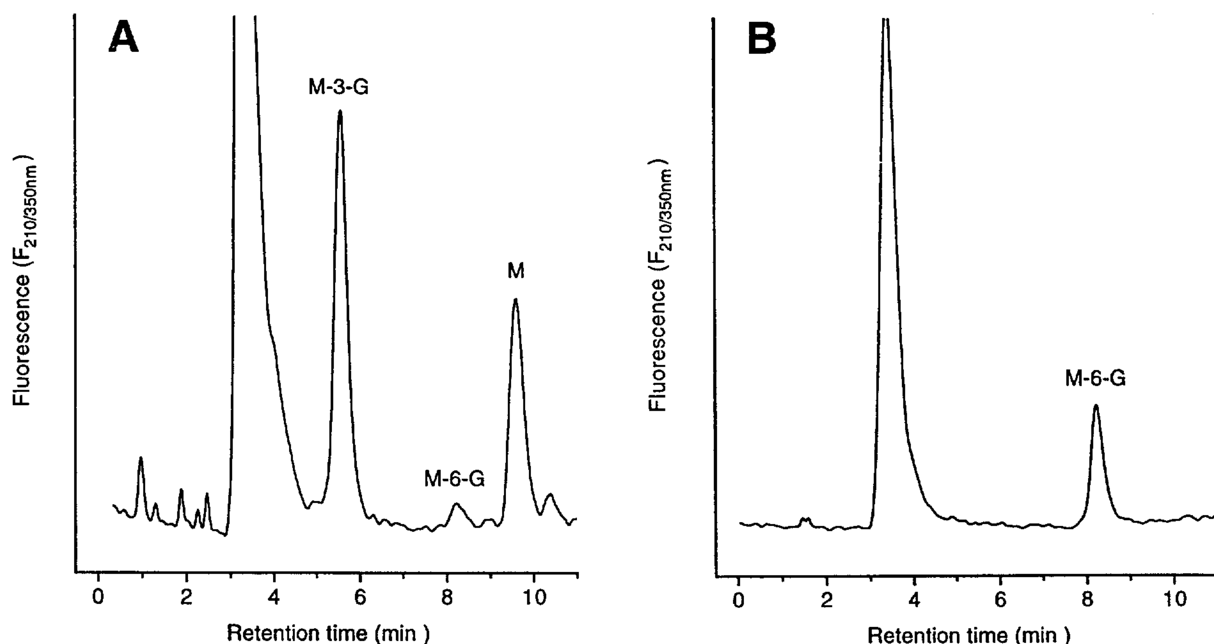

Fig. 2. (A) Representative chromatogram of a blood sample drawn 1 h after intravenous infusion of 10 mg morphine containing 12.8 ng/ml M-3-G, 3.4 ng/ml M-6-G and 8.3 ng/ml morphine. Sample size used for analysis was 400 μl. (B) Representative chromatogram of a blood sample drawn 1 h after intravenous infusion of 16 mg M-6-G containing 250 ng/ml M-6-G. Sample size was 100 μl.

Table 2
Accuracy and intra- and inter-day assay variability for morphine-3-glucuronide (M-3-G), morphine-6-glucuronide (M-6-G) and morphine

Compound	Nominal concentration (ng/100 μl)	Concentration found (mean ± S.E.M., $n = 4$) (ng/100 μl)	Accuracy[a] (%)	Assay variability (C.V., %)	
				Intra-day ($n = 4$)	Inter-day ($n = 4$)
M-3-G	0.50	0.58 ± 0.27	15.1	8.9	12.5
	2.0	2.13 ± 0.19	6.3	4.5	8.5
	10.0	10.25 ± 0.19	2.5	1.4	5.3
	20.0	19.69 ± 0.18	−1.5	1.0	3.0
	50.0	49.64 ± 0.60	−0.7	0.5	2.3
	100.0	98.49 ± 1.40	−1.5	1.4	2.3
M-6-G	0.50	0.40 ± 0.12	−19.8	6.8	15.4
	2.0	1.94 ± 0.06	−3.2	6.2	8.5
	10.0	10.17 ± 0.24	1.7	2.8	5.7
	20.0	19.86 ± 0.15	−0.7	1.3	2.9
	50.0	50.25 ± 0.06	0.5	0.6	1.8
	100.0	99.89 ± 0.04	−0.1	1.9	2.1
Morphine	0.50	0.45 ± 0.04	−9.4	9.1	13.3
	2.0	1.96 ± 0.02	−2.2	4.3	7.4
	10.0	10.18 ± 0.22	1.8	1.0	5.2
	20.0	19.83 ± 0.10	−0.8	1.3	2.6
	50.0	50.13 ± 0.25	0.3	0.1	1.9
	100.0	99.95 ± 0.11	−0.01	0.3	2.1

[a] Defined as the percentage deviation between the mean concentration found and the theoretical concentration.

a given amount of substance (20 ng) applied in 100 μl or 400 μl of plasma ($n=4$; Student's t-test, $p>0.1$ for all compounds). Using injection volumes of 400 μl our system allows to detect 0.85, 3.4 and 1.0 ng/ml of M-3-G, M-6-G and morphine, respectively, in plasma. These figures were determined in a separate set of experiments using injection volumes of 400 μl. Further improvement of the detection limit may be possible while using larger extraction cartridges and/or larger sample volumes.

3.4. Quality control assessment

The intra- and inter-assay variability was consistent over a wide concentration range (2–100 ng/ml of morphine and its glucuronides in plasma), with coefficients of variation (C.V.) values being less than 10% (Table 2). Even inter-day assay variability for amounts of substances representing or being close to the detection limit of the system were not higher than 15.4% (Table 2).

3.5. Assay application

The assay presented here has been used to study the pharmacokinetics and pharmacological potency of morphine and its metabolites in man. Healthy volunteers were given intravenous infusion of morphine or morphine glucuronides. Blood samples were drawn at defined time points for the determination of morphine and its glucuronides. Representative chromatograms of blood samples taken 1 h after start of the intravenous infusion of 10 mg morphine (Fig. 2A) or an equimolar amount of M-6-G (Fig. 2B) are shown.

4. Conclusion

A HPLC-method has been described which allows fully automated and rapid determination of morphine and morphine glucuronides in plasma or cerebrospinal fluid. In combination with its sensitivity and the small sample size required, the assay presented here may be easily used for extensive in vivo or in vitro pharmacological studies or for routine analysis of body fluids of patients being treated with morphine.

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